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The developmental stage of inactivation of rye origin rRNA genes in the embryo and endosperm of wheat × rye F_1 hybrids

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To identify the developmental stage during which the preferential inactivation of rRNA genes from the rye parent occurs in wheat × rye hybrids, nucleolar activity was evaluated in the embryo and endosperm of developing seeds of the hybrids. The hybrids were obtained from crosses of euploid and aneuploid lines of hexaploid wheat, *Triticum aestivum* cv. Chinese Spring, with rye, *Secale cereale* cv. Centeio do Alto. The number of nucleolar organizing regions (NORs) and nucleoli present in the embryo and endosperm cells of wheat, and wheat × rye F_1 hybrids, at different times after fertilization was scored by silver staining. The inactivation of rDNA of rye origin in F_1 hybrids occurs simultaneously in the embryo and in the endosperm between 4 and 5 days after fertilization, when these have been through six and 10 cell cycles respectively. We conclude that the genomic interactions leading to the inactivation of the rye origin rDNA is a time-dependent process, related to the developmental stage and independent of the number of cell cycles (DNA replication rounds) they have been through.

Key words: genomic imprinting, intergenomic interactions, 1R nucleolar organizer region, seed development

Introduction

In bread wheat, *Triticum aestivum* (L.) em. Thell., ribosomal RNA (rRNA) genes are found in four major nucleolar organizing regions (NORs) on chromosome pairs 1B and 6B, accounting for 90% of total cell rDNA (Flavell & O'Dell 1976), and in six minor NORs located on chromosome pairs 5D and 1A (Crosby 1957, Darvey & Driscoll 1972, Viegas & Mello-Sampayo 1975, Flavell & O'Dell, 1976, Martini & Flavell 1985) and chromosome 7D (Mukai *et al.* 1991), but there is no evidence for expression of the latter. The genes have been named NOR-B1, NOR-B2, NOR-D3, NOR-A and NOR-D4 (McIntosh 1993).

These NORs show differential activity, with 1B NORs twice as active as 6B NORs, judging by the volume of the nucleolus they produce (Martini & Flavell 1985), although 1B NORs have half the number of rDNA units present in the 6B loci (Flavell & O'Dell 1976). The physical basis of wheat intraspecific nucleolar dominance has been suggested to be related to the undermethylation of cytosine residues, with dominant or partially dominant loci showing reduced methylation in comparison with inactive loci, as changes in cytosine methylation could be responsible for alterations in the chromatin structure of the promoter regions of rRNA genes (Flavell *et al.* 1988, Flavell 1989). Similarly, when chromosomes of related species, carrying NORs, are added to wheat, interspecific nucleolar dominance – amphiplasty – can usually be detected (Rieger *et al* 1979). In wheat × rye hybrids, nucleolar dominance of wheat is observed, resulting in an almost total inactivation of the rRNA genes of rye (*Secale cereale* L.) origin, located on chromosome 1R (Appels *et al* 1986, Gustafson *et al* 1988, Silva *et al* submitted). The 1R NOR inactivation has also been shown to correlate with extensive methylation of DNA since it is possible to induce its expression by the incorporation of 5-azacytidine, a methylation-inhibiting analog, in the newly replicating DNA of root tip cells of wheat × rye F_1 hybrids (Vieira *et al.* 1990) and triticale (Heslop-Harrison 1990).

Rye meiotic cells carry an active 1R NOR, but the 1R NOR is inactive in root tips cells of germinating wheat × rye F_1 hybrid seeds. Silver staining (Schwarzacher *et al.* 1978, Hubbel 1985, Jiminez *et al.* 1988) shows nucleoli within interphase nuclei and chromosomes with NORs which were transcriptionally active at the previous interphase. In this paper, we aimed to identify the stage of development when inactivation of the rye 1R NOR occurs in wheat × rye hybrids by silver staining developing embryos and endosperms at different times after fertilization.

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Materials and methods

The following plants from the North of Portugal were grown in the greenhouse: (i) bread wheat *Triticum aestivum* (L.) em. Thell cv. Chinese Spring ($2n = 6x = 42$, genome designation AABBDD); (ii) aneuploid nullisomic–tetrasomic (N–T) derivatives from hexaploid wheat in which a pair of homologous chromosomes is replaced by an additional pair of homologous chromosomes (from stocks kindly supplied by the late Dr E. R. Sears); and (iii) *Secale cereale* L. cv. Centeio do Alto ($2n = 2x = 14$, genome designation RR). Euploid and aneuploid wheat plants were emasculated, retaining only the primary and secondary florets, and pollinated with either wheat or rye pollen. After 3–7 days, pollinated plants were transferred to a cold chamber at 0–2°C for 24 h to accumulate C-metaphases and shorten chromosomes. After the cold treatment the ovaries of each spike were excised and fixed in FAA [a mixture of 37% (v/v) formaldehyde, glacial acetic acid and 50% (v/v) ethanol, 1:1:18] for 2–3 days at 4°C. To stain NORs and nucleoli selectively, a silver nitrate impregnation technique was used. Briefly, ovaries were thoroughly washed with distilled water to remove fixative solution and treated in a 20% (w/v) silver nitrate solution at 60°C in the dark overnight. To develop the silver nitrate staining, the ovaries were washed several times and transferred into a 1:1 (v/v) 10% formaldehyde–1% hydroquinone solution for 5 min at room temperature. The ovaries were then washed and immersed in a photographic fixative solution [stock solution, 25% (v/v) Kodafix in distilled water, stored at 4°C; working solution, fresh 25% (v/v) Kodafix–dH_2O in a 1:7 proportion] to prevent further reaction. Ovules were further dissected from each ovary in 45% (v/v) acetic acid and the embryo and endosperm pulled apart after the removal of the ovule wall. The embryo and the endosperm of each ovule were squashed separately in 45% (v/v) acetic acid and the number of NORs in metaphase cells and the number of nucleoli in interphase cells scored. The number of cells in the embryos and endosperms was also recorded.

Results and discussion

Wheat × rye F_1 hybrids from wheat euploid genotypes

Table 1 shows the range of total cell numbers in 3- to 7-day-old embryos (diploid cells) and endosperms (triploid cells) of wheat seeds and in wheat × rye F_1 hybrids, an example of which is shown in Figure 1. In our greenhouse conditions, we expect fertilization to occur during the first hour after pollination (see Bennett *et al.* 1973). No differences were observed in rates of development of different seeds so the ontogenic stage of seed development will be referred to only by the number of days after pollination. These rates may be slightly different from those obtained by other authors (Bennett *et al* 1975), reflecting the different environmental conditions during development.

Table 2 presents the number of C-metaphase cells with each number of silver-stained NORs (Ag-NORs) and the frequencies of interphase cells with different numbers of nucleoli in embryos and in endosperms of wheat and of wheat × rye F_1 hybrids 3–7 days after pollination.

Embryos

In both 3- and 7-day-old embryos of bread wheat, four Ag-NORs were always observed; these can be ascribed to chromosomes 1B and 6B (Flavell & Smith 1974, Flavell & O'Dell 1976) since minor NORs, located in chromosomes 1A and 5D, although sometimes transcribing visible nucleoli, are rarely detectable by silver staining on metaphase cells.

In wheat × rye F_1 hybrids three Ag-NORs, belonging to wheat chromosomes 1B and 6B and to rye chromosome 1R, were consistently found in all metaphase cells

Table 1. The cell number in embryos and endosperms during the first days of seed development in common wheat and wheat × rye F_1 hybrids

Days after pollination	Range of total number of embryo endosperm and cells			
	Common wheat		Wheat × rye F_1 hybrids	
	Embryo (AABBDD)	Endosperm (AAABBBDDD)	Embryo (ABDR)	Endosperm (AABBDDR)
3	4–5	150–200*	7–8	300–400*
4	—	—	22–30	550–670*
5	—	—	45–52	>1000
6	—	—	100–150	>3000
7	150–200	>5000	200–350	>5000

*Numbers refer to nuclei rather than to cells since the endosperm is coenocytic at these ages.

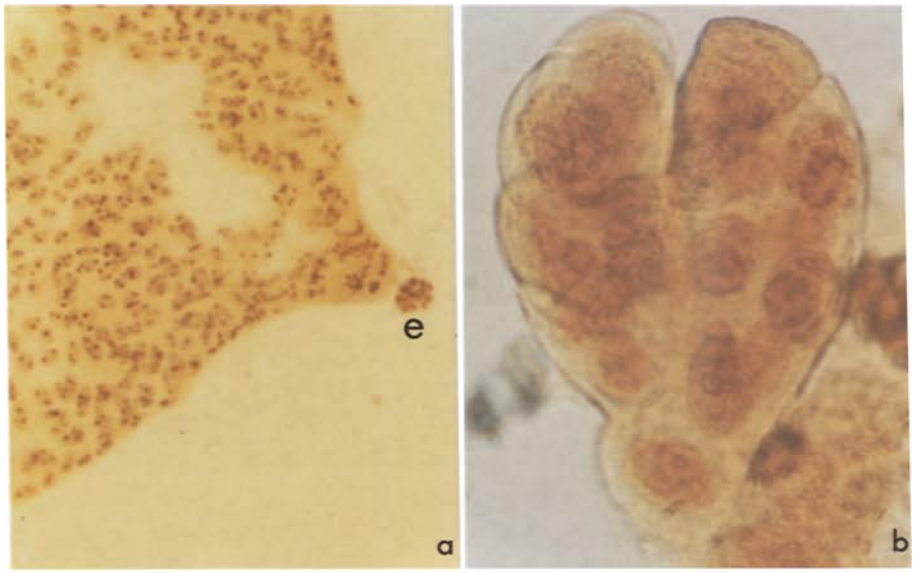

Figure 1. Dissected ovule of a wheat × rye F_1 hybrid after 5 days of pollination, showing **a** the endosperm and the relative position of the embryo (e) and **b** a particular aspect of the embryo.

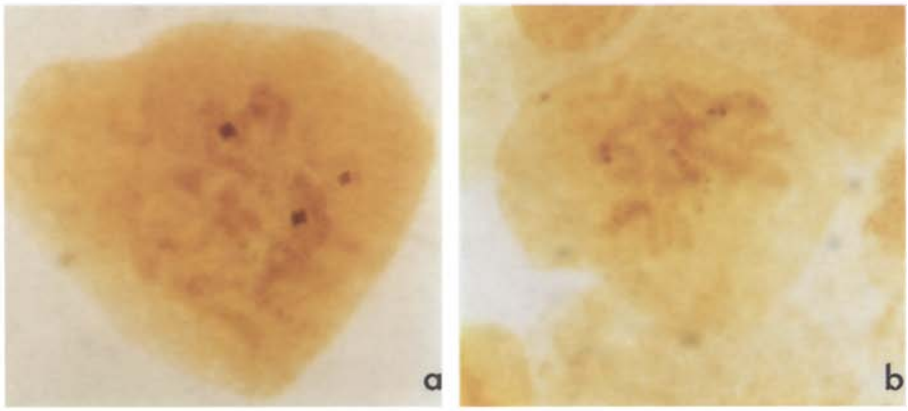

Figure 2. Embryo metaphase cells from a wheat × rye F_1 hybrid, showing the number of Ag-NORs present. **a** A 4-day-old embryo presents three Ag-NORs and **b** a 5-day-old embryo presents two Ag-NORs.

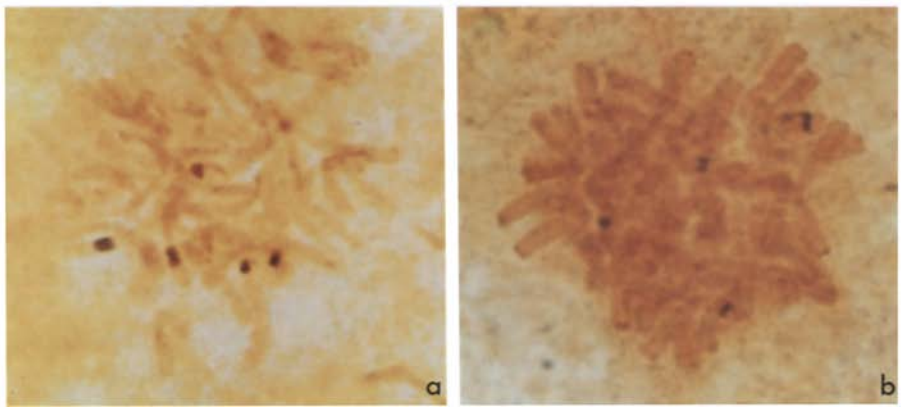

Figure 3. Endosperm metaphase cells from a wheat × rye F_1 hybrid, showing the number of Ag-NORs present. **a** A 5-day-old endosperm presents five Ag-NORs and **b** a 4-day-old endosperm presents four Ag-NORs.

of 3- and 4-day-old embryos, but only two Ag-NORs were present in all embryos 5–7 days after pollination (Figure 2a & b respectively). The maximum number of nucleoli observed in the interphase cells of the same material also reveals a similar reduction from a maximum of four nucleoli in 3- and 4-day-old embryos to a maximum of three nucleoli in the older ones. In fact, NORs become active and form nucleoli after cell division. The nucleoli normally fuse as the cell cycle advances, so the maximum number regularly observed in silver-stained preparations indicates the number of active NORs.

Table 2. Number of Ag-NORs in C-metaphase cells and frequency of interphase cells with different numbers of nucleoli in embryos and endosperms of wheat × rye F_1 hybrids and bread wheat 3–7 days after pollination

Material	Genotype	Days after pollination	No. of C-metaphase cells with different nos. of NORs						Frequency of interphase cells with different numbers of nucleoli (%)									No. of cells observed	No. of developing seeds observed
			2	3	4	5	6	7	1	2	3	4	5	6	7	8	9		
Embryo	*T. aestivum* ×	3	—	9	—	—	—	—	26.14	46.47	25.73	1.66	—	—	—	—	—	241	24
		4	—	5	—	—	—	—	33.97	44.98	20.57	0.48	—	—	—	—	—	209	11
	S. cereale	5	8	—	—	—	—	—	32.43	55.72	11.85	—	—	—	—	—	—	1249	30
	(ABDR)	6	6	—	—	—	—	—	30.07	56.27	13.66	—	—	—	—	—	—	542	8
		7	21	—	—	—	—	—	27.94	56.84	15.22	—	—	—	—	—	—	1965	18
	T. aestivum	3	—	—	6	—	—	—	—	36.17	50.06	12.37	1.40	—	—	—	—	47	10
	(AABBDD)	7	—	—	4	—	—	—	6.05	23.02	37.67	30.70	2.53	—	—	—	—	430	4
Endosperm	*T. aestivum* ×	3	—	—	11	228	13	10	3.04	16.63	31.84	29.41	15.88	2.76	0.28	0.06	—	8042	42
		4	—	—	9	203	5	—	3.32	19.65	36.24	28.03	10.55	1.83	0.36	0.02	—	4484	28
	S. cereale	5	—	—	122	5	—	—	5.08	29.70	38.38	20.97	5.29	0.54	0.04	—	—	5179	24
	(AABBDDR)	6	—	—	72	5	—	—	3.55	21.39	38.10	28.72	7.26	0.83	0.15	—	—	1323	9
		7	—	—	162	2	—	—	3.16	14.82	31.78	31.74	14.89	3.58	0.63	—	—	1424	10
	T. aestivum	3	—	—	—	3	228	5	0.47	4.39	16.53	25.58	23.99	15.97	8.78	4.20	0.09	1071	13
	(AAABBBDDD)	7	—	—	—	—	219	—	0.12	2.30	10.30	25.82	33.58	18.55	7.51	1.58	0.24	825	8

Table 3. Maximum number of Ag-NORs in C-metaphase cells and average number of nucleoli in interphase cells of embryos and endorsperms from wheat aneuploids × rye F_1 hybrids, 4 and 5 days after pollination

Material	Wheat genotype[a]	No. of days after pollination	Maximum no. of Ag-NORs regularly observed	No. of cells observed	Maximum no. of nucleoli regularly observed	No. of cells observed
Embryo	N1AT1B	4	—	—	5	176
		5	—	—	4	488
	N5BT5D	4	3	1	4	160
		5	2	2	4	150
	N6BT6A	4	2	1	3	15
		5	1	3	3	204
Endosperm	N1AT1B	4	—	—	6	1176
		5	6	131	5	1046
	N6BT6A	4	3	96	7	507
		5	2	34	6	498
	N5BT5D	4	6	12	5	683
		5	5	116	5	959

[a]aN1AT1B and similar nomenclature = nullisomic for 1A and tetrasomic for 1B.

Comparison of the number of Ag-NORs present in the embryo cells of wheat × rye F_1 hybrids shows an additional active NOR, belonging to chromosome 1R, until the fourth day after pollination while this extra NOR is no longer active in embryos on days 5–7 after pollination (Figure 2b). As in mature seeds of wheat × rye F_1 hybrids and of the artificial amphiploid triticale, the rye NOR is inactive in most cells (Lacadena *et al* 1984), so the reduction in major Ag-NOR numbers observed between the fourth and the fifth days after pollination must reflect inactivation of the NOR from rye origin. The 1R NOR inactivation must therefore occur when the embryo has approximately 35 cells (*i.e.* after 5–6 cell cycles, Table 1). This inactivation process is probably not complete, since a slight activity was detected by silver staining technique (Silva *et al.* submitted) and by *in situ* hybridization (Appels *et al.* 1986, Gustafson *et al.* 1988). Such activity of the 1R NOR must be too low to produce visible nucleoli since we also observed a reduction in the maximum number of nucleoli. The allocation of this extra Ag-NOR to chromosome 1R and not to chromosomes 1A or 5D is based on chromosome morphology as the three visible Ag-NORs observed in embryo cells until the fourth day after pollination are all subterminal and located in chromosomes with marked satellites, corresponding to chromosomes 1B, 6B and 1R.

Endosperm

In wheat endosperm most of the cells have six major NORs regardless of the developmental stage analysed, and the low activity of minor NORs was similar to that in meristematic root tip cells (Silva *et al* submitted).

In the endosperm cells of the wheat × rye F_1 hybrids the inactivation of the 1R NOR was found to follow the pattern of inactivation described for the embryo cells: the number of major NORs detected by silver staining in the endosperm cells of the hybrid is reduced from 5 to 4 on the fifth day after pollination (Figure 3a & b respectively), corresponding to the four major wheat NORs present. A reduction of the maximum number of nucleoli from 8 to 7 was observed when analyzing their distribution in interphase cells, confirming the previous results.

The inactivation of the 1R NOR in the endosperm occurs after 9–10 cell cycles, when it has around 800 cells (Table 1), suggesting that the gene and genomic interactions leading to this amphiplasty phenomenon, the 1R NOR inactivation in a wheat background, occurs simultaneously in both embryo and endosperm, being independent of the cell division rate.

Wheat × rye F_1 hybrids from wheat aneuploid genotypes

Further analysis of some F_1 hybrids derived from wheat aneuploid genotypes with different numbers of minor (1A and 5D) and major (1B and 6B) NORs was performed. The results, summarized in Table 3, show a decrease in the number of silver-stained NORs and nucleoli both in the endosperm and in the embryo between the fourth and fifth day of seed development. The trend is independent of the number of major and minor wheat NORs present in each genotype so the residual expression of 1R NOR is not changed when one major wheat NOR is deleted. Thus the wheat NORs (minor and remaining major) probably increase their activity to compensate for the loss of a major NOR, as discussed by Martini & Flavel (1985).

The results presented here also indicate that some genes involved in the 1R NOR inactivation are not located on wheat nucleolar chromosomes as previously observed in meristematic cells of aneuploids wheat × rye F_1 hybrids (Vieira *et al.* 1990).

Conclusions

We show that the process of 'genomic imprinting' in the wide sense, which is responsible for nucleolar dominance in wheat × rye hybrids, is imposed between 4 and 5 days after fertilization, after which the rDNA expression pattern is maintained. Interestingly, the timing of the 1R NOR inactivation during early seed development is not dependent on the number of DNA replication rounds as it occurs simultaneously in the endosperm and embryo when they have been through 10 and five cell divisions respectively.

DNA methylation has been found to be actively involved in the mechanism of nucleolar dominance in the Triticeae, leading to a hierarchy of NOR expression (Heslop-Harrison 1990, Vieira *et al.* 1990, Neves *et al.* submitted).

The process responsible for the preferential inactivation of rDNA loci in some Triticinae hybrids can be considered analogous to the one responsible for the changes in DNA methylation observed during animal gametogenesis and embryogenesis, mediating several parental imprinting phenomena and X-chromosome inactivation respectively (Monk *et al.* 1987, Ferguson-Smith & Surani 1993).

The precise mechanisms involved in the regulation of preferential rDNA loci inactivation are not yet fully understood (Appels 1986, Flavel 1989). The allocation of that phenomenon to a specific developmental stage will contribute to further detailed molecular and developmental scrutiny, leading therefore to a better understanding of genomic interactions in plant.

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